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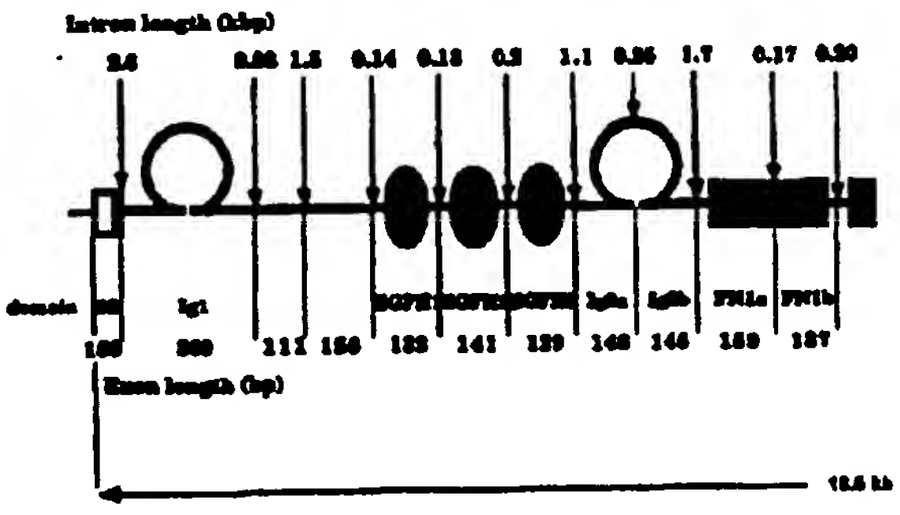
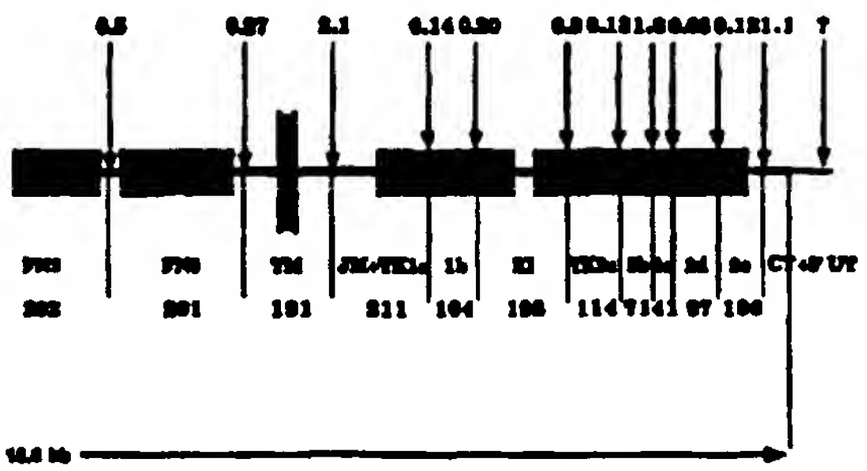
(54) Title: PROMOTER FOR THE RECEPTOR TYROSINE KINASE, TIE

(57) Abstract

The present application discloses promoter sequences for *Tie*, an endothelial cell receptor tyrosine kinase and their use in therapy and diagnosis as well as production of proteins in blood and tissues.





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PROMOTER FOR THE RECEPTOR TYROSINE KINASE, *TIE*

FIELD OF THE INVENTION

The present invention relates generally to receptor tyrosine kinases and promoters thereof.

BACKGROUND OF THE INVENTION

The circulatory system is the first organ system to differentiate in the developing embryo. Kaufman, The Atlas of Mouse Development, Academic Press, (1992). Embryonic and yolk sac vascular systems take form in an 8.5 day p.c. mouse embryo and a day later the heart beats regularly, circulating primitive blood cells, nutrients, and metabolic waste products. Endothelial cells covering blood vessels provide a barrier between blood and other tissues of the embryo. When organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases. Fenestrated vessels, nonfenestrated vessels with tight junctions and sinusoidal vessels are found, for example, in the kidney, brain, and liver, respectively. In addition, endothelial cells perform specific functions in differentiated tissue. For example, such cells take part in several biochemical and physiological events such as blood cell trafficking, blood clotting, hemostasis, ovulation, wound healing, atherosclerosis, and angiogenesis associated with tumor metastasis.

At least five receptor tyrosine kinase genes are expressed in endothelial cells. Of these, the protein products of the FLT1, KDR/FLK-1, and FLT4 genes belong to receptor tyrosine kinase subclass III; whereas *Tie* and its close relative Tek

(*Tie*-2) form a novel subclass of their own (Terman, *et al.*, *Oncogene*, *6*: 1677-1683, 1991, Terman, *et al.*, *Biochem. Biophys. Res. Comm.*, *187*: 1579-1586, 1992, Aprelikova, *et al.*, *Cancer Res.*, *52*: 746-748, 1992, De Vries, *et al.*, *Science*, *255*: 989-991, 1992, Pajusola, *et al.*, *Cancer Res.*, *52*: 5738-5742, 1992, Sarzani, *et al.*, *Biochem. Biophys. Res. Comm.*, *186*: 796-714, 1992, Galland, *et al.*, *Oncogene*, *8*: 1233-1240, 1993, Millauer, *et al.*, *Cell*, *72*: 835-846, 1993, Oelrichs, *et al.*, *Oncogene*, *8*: 11-18, 1993, Schnurch and Risau, Development, 119: 957-968, 1993). Both human and mouse *Tie* cDNAs have been cloned (Partanen, *et al.*, *Mol. Cel. Biol.*, *12*: 1698-1707,1992, Korhonen, *et al.*, *Blood*, *80*: 2548-2555, 1992, Korhonen, *et al.*, *Oncogene*, *8*: 395-403, 1994, Iwama, *et al.*, *Biochem. Biophys. Res. Comm.*, *195*: 301-309, 1993, Sato, *et al.*, *Proc. Natl. Acad. Sci. USA.*, *90*: 9355-9358, 1993). *Tie* and homologous genes have been isolated from bovine and rat sources (Maisonpierre, *et al.*, *Oncogene*, *8*: 1631-1637,1993, Sato, *et al.*, *Proc. Natl. Acad. Sci. USA.*, *90*: 9355-9358, 1993). Genomic clones for mouse *Tie* and both mouse and human *Tie* promoter regions have been cloned and characterized.

The 4.4 kb *Tie*-encoding mRNA encodes a 125 kDa transmembrane protein which is N-glycosylated. In its extracellular domain *Tie* contains two immunoglobulin-like loops and three epidermal growth factor and fibronectin type III homology regions, which are followed by trans- and juxtamembrane domains connected to a tyrosine kinase domain which is split by a short kinase insert sequence and a carboxyl terminal tail (Partanen, *et al.*, *Mol. Cel. Biol.*, *12*: 1698-1707, 1992, Korhonen, *et al.*, *Oncogene*, *8*: 395-403, 1994, Sato, *et al.*, *Proc.*

Natl. Acad. Sci. USA., 90: 9355-9358, 1993). Both *Tie* and TEK have been localized to mouse chromosome 4 at a distance of 12.2 cm from each other. Such receptors are uniformly expressed in endothelial cells of various blood vessels during embryonic development, although the expression of Tek mRNA appears to begin 0.5 days earlier than the expression of *Tie*. In adult mice, the expression of *Tie* mRNA persists in vessels of the lung whereas in the heart and brain it appears to decrease. Korhonen, *et al., Oncogene, 8*: 395-403, (1994). Production of *Tie* mRNA is enhanced during ovulation and wound healing and in human glioblastomas (Korhonen, *et al., Blood, 80*: 2548-2555, 1992).

Endothelial cells play a key role in gene therapy directed to diseases involving endothelial cells and blood vessels, such as establishment of neovascularization or inhibition of angiogenesis, and control of inflammatory trafficking of leukocytes. One approach to the treatment of vascular disease is to express genes at specific sites in the circulation that might ameliorate the disease *in situ*. Because endothelial cells are found at diseased sites, they represent logical carriers to convey therapeutic agents that might include anticoagulant, vasodilator, angiogenic or growth factors. Accordingly, the genetic modification of endothelial cells represents a therapeutic approach to the treatment of many vascular disorders, including hypertension, atherosclerosis and restenosis. For example, endothelial cells expressing growth inhibitory proteins could be introduced via catheter to the angioplasty site to prevent local intimal hyperplasia and clinical restenosis. The luminal

surface of vascular grafts could also be lined with genetically modified endothelial cells producing therapeutic proteins which prevent thrombosis or promote repopulation (Nabel, *et al.*, J. Am. Coll. Cardiol., *17*, 189B-194B, 1991).

Endothelial cells lining blood vessels are easily transfected with methods using liposomes, adenovirus vectors and retroviral vectors (Nabel, *et al.*, *J. Am. Coll. Cardiol. 17*: 189B-94B). Endothelial cells are also in direct contact with blood and are therefore optimal sources for production and secretion of desired proteins or peptides into the blood stream. For example, the Factor VIII gene may be introduced into endothelieal cells under an endothelial cell- specific promoter, resulting in correction of hemophilia if the protein were expressed in sufficeint quantity. On the other hand, endothelial cells are also useful for delivery of peptides or proteins expressed in them into tissues. In this regard, a selective expression of a particular gene regulatory element in endothelial cells of the microvasculature (capillaries) is extremely useful, given that most of the cell surface area facing the vascular lumen consists of microvascular endothelial cells.

Control elements of the endothelial cell specific promoters may be further subdivided and dissected into functional elements and units according to methods standard in the art. The *Tie* protein is expressed in certain endothelial cells and about 0.9% of human bone marrow cells. Therefore, it is likely that the *Tie* promoter is active also in some hematopoietic cells. However, expression of the *Tie* promoter in hematopoietic cells may be controlled by elements which are

distinguishable from endothelial- cell-specific elements and may be dissected away while retaining the endothelial cell specificity of the promoter.

The present invention provides a novel promoter associated with the gene encoding the *Tie* receptor tyrosine kinase for use in therapeutic and diagnostic procedures. In addition, the promoter may prove useful in the production of desired proteins to the blood or tissues of animals.

SUMMARY OF THE INVENTION

The present invention generally relates to promoter sequences for the receptor tyrosine kinase, *Tie*. In a preferred embodiment of the invention, a mouse *Tie* promoter is provided comprising the sequence shown in SEQ ID NO:1. Also in a preferred embodiment, a human *Tie* promoter is provided comprising the sequence shown in SEQ ID NO:2. A promoter according to the invention drives the expression of endothelial cell receptor tyrosine kinases, and in particular, the receptor tyrosine kinase, *Tie*.

A vector according to the present invention may be any vector suitable for incorporating a promoter according to the invention and may preferably be the 5.0hpromSDKLacZ vector deposited on September 20, 1994 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, as Accession Number 75892. Host cells according to the invention may be any host cell capable of housing the promoter or a vector containing the promoter according to the invention. Examples of host cells according to the invention are LEII endothelial cells.

Other advantages and uses of the invention will be apparent upon consideration of the following Detailed Description thereof.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the mouse *Tie* gene and promoter.

Figure 2 is a comparison of mouse and human *Tie* promoter sequences.

Figure 3 shows results of an analysis of *Tie* promoter activity.

Figures 4A shows expression patterns of the *Tie* promoter in the developing endocardium and head mesenchyme of 8.5 day mouse embryos.

Figure 4B show expression of a mouse *Tie* promoter construct in yolk sac blood islands in 8.5 day embryos.

Figure 5A and 5B show the expression pattern of mouse *Tie* promoter in 9.5 day embryos.

Figures 5C and 5D show the expression pattern of mouse *Tie* promoter in 11.5 day embryos.

Figure 6A shows expression of the *Tie* promoter in 9.5 day embryonic heart tissue.

Figure 6B shows expression of the *Tie* promoter in 11.5 day embryonic lung tissue.

Figure 6C shows expression of the *Tie* promoter in 15.5 day embryonic brain tissue.

Figure 6D shows expression of the *Tie* promoter in 15.5 day embryonic liver tissue.

Figure 6E shows expression of the *Tie* promoter in developing bone trabeculae.

Figure 6F shows expression of the *Tie* promoter in developing kidney tissue.

Figure 7A shows expression of the *Tie* promoter in the interalveolar capillaries of the lung in an 8-week-old mouse.

Figure 7B shows expression of the *Tie* promoter in the endothelial network of the bone marrow in an 8-week-old mouse.

Figure 7C shows expression of the *Tie* promoter in kidney tissue of an 8-week-old mouse.

Figure 7D shows expression of the *Tie* promoter in heart tissue of an 8-week-old mouse.

Figure 7E shows expression of the *Tie* promoter in liver tissue of an 8-week-old mouse.

Figure 7F shows expression of the *Tie* promoter in brain tissue of an 8-week-old mouse.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides promoter sequences capable of directing the expression of recombinant DNA sequences in endothelial cells. In particular, the invention provides promoter sequences which direct expression of the beta-galactosidase reporter gene in endothelial cells of mouse tissues. Promoters for production of proteins and peptides which act as anticoagulants, vasodilator inhibitors of thrombosis or restenosis into endothelial cells, blood and tissues. Promoters according to the present invention are useful for directing expression of proteins and peptides for human gene therapy, antigens and markers useful for endothelial cell tagging, and antisense RNA constructs for use in endothelial cells *in vivo* and *in vitro*. Promoters, vectors, and host cells according to the invention are also useful in gene therapy for promoting expression of various growth factors or receptors or their

domains. Moreover, analogs of promoters according to the invention are useful for inhibiting undesired endothelial cell proliferation as, for example, the inhibition of angiogenesis during tumor formation.

EXAMPLE I

Cloning and characterization of the genomic *Tie* DNAs.

A. Mouse Genomic *Tie*

In order to characterize the genomic organization of the mouse *Tie* gene, approximately 3 x 10^6 plaques were screened. The plaques were obtained from a genomic library made from DNA of adult SV129 mouse liver cells (Clontech) using as a probe a mouse 1C1D cDNA fragment (Korhonen, *et al.*, *Blood.*, *80*:2548-2555, 1992) encoding the epidermal growth factor homology domains [GCVKDCPGCLHGGVCHDHDGCVCPPGFTGTRCEQACREGRFGQSCQEQCPG TAGCRGLTFCLPDPYGCSCGSGWRGSQCQEACAPDHFGADCRLQCQCQNGGT CDRFSGCVCPSGWHGVHCEKSDRIPQIL: SEQ ID NO:3] Three separate clones, SV1, SV2, and m*Tie* were obtained thereby and each was subcloned into pGEM 3Zf(+) (Promega) and characterized by partial dideoxy chain termination sequencing and restriction enzyme analysis. A schematic structure of the mouse *Tie* gene and its promoter is shown in Figure 1. In that Figure, the positions of introns are indicated by arrows and their lengths are indicated. Restriction mapping, PCR, and nucleotide sequence analysis showed that the *Tie* gene spans approximately 19 kb of genomic DNA. *Tie* is encoded by 23 exons. The distinct structural domains of the extracellular portion are encoded by either one exon each, comprising the first immunoglobulin-like loop, epidermal growth factor homology domains 1-3 and

fibronectin-like domains 2 and 3, or by two exons comprising the second immunoglobulin-like loop and first fibronectin-like domain. The transmembrane region is encoded by a distinct exon; whereas the tyrosine kinase domain containing the kinase insert is encoded by eight exons of which the first encodes the juxtamembrane region. The lengths of the introns vary from 80 bp to 2.6 kb.

B. Human Genomic *Tie*

Three human *Tie* clones were isolated from a human placental cDNA library in the EMBL-3 vector system (Clontech) as shown in Partanen, *et al.*, *Mol. Cell. Biol.*, *12*: 1698-1707 (1992); incorporated by reference herein. To obtain the human *Tie* clones, a PCR fragment encoding the *Tie* signal sequence was amplified from human *Tie* cDNA using the primers, 5'-CCCACATGAGAAGCC-3' (SEQ ID NO: 4) and 5'-TGAGATCTTGGAGTATGGTCTGGCGGGTGCCC-3' (SEQ ID NO: 5), and used to probe the aforementioned library. The resulting positive clone containing the longest insert was plaque-purified and an approximately 7 kb *Sac*I fragment was subcloned in pGEM 3Zf(+) and characterized. The resulting human *Tie* promoter sequence is shown in Figure 2. In that Figure, transcription initiation sites are marked with an asterisk (See primer extension and RNAse protection experiments below). Restriction endonuclease cleavage sites discussed herein are marked in bold.

A comparison of the genomic DNA sequences of mouse and human *Tie* promoters is also shown in Figure 2. In that Figure, the mouse sequence extends from the 3' end of the first exon to the *Afl*II site which is approximately 772 bp upstream

from the ATG codon. A CA repeat found only in the mouse sequence is highlighted in bold in Figure 2.

EXAMPLE II

Determination of The Transcription Initiation Site in The Human *Tie* Gene

For primer extension analysis of *Tie*-encoding nucleic acids, primer was labelled according to the manufacturer's instruction (Promega, USA). An aliquot of 10 pmol primer was then incubated with 10 x forward exchange buffer (Promega), 10 mCi/ml [g-32p]-ATP, and 10U T4 polynucleotide kinase at 37°C for 1 hour. The kinase was then inactivated by heating at 90°C for 2 minutes and the labelled primer was ethanol precipitated.

Poly (A+) RNA (20 mg) and 5 x 10^5 cpm labelled primer were then annealed in hybridization buffer (40 mM PIPES pH 6.4, 1mM EDTA pH 8.0, 0.4 M NaCl and 80% formamide) by heating at 95°C for 12 minutes. Samples were then cooled slowly and ethanol precipitated. The resulting dried annealing mixture was suspended in primer extension buffer (50 mM Tris- HCl, 50 mM KCl, 10 mM MgCl2, 10 mM DTT, 2 mM each of deoxy ATP, deoxy CTP, deoxy GTP, and deoxy TTP, 0.5 mM spermidine, pH 8.3, at 42°C) and 20 U RNAsin and 40 U AMV reverse transcriptase were added. After 2 hours of incubation, template RNA was digested by addition of 20 mg/ml RNAse A in 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, 37°C for 15 minutes. The resulting mixture was phenol extracted and ethanol precipitated. The pellet was then resuspended in loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 1% bromophenol blue) and loaded onto a 9% polyacrylamide/7M urea gel. After

electrophoresis, the dried gels were exposed to x-ray film for 2 days.

RNAase protection was accomplished using mouse RNA antisense probes of 291 bp and 239 bp generated from linearized plasmids containing the 754 bp *Afl*II - *Bam*HI and 1.1 kb *Hind*III - *Apa*I mouse *Tie* promoter DNA inserts. The human RNA probe of 568 bp was generated from linearized pGEM 3Zf(+) plasmid (Promega, USA) containing an *Acc*I-AlwNI human *Tie* promoter DNA insert. The template for the other human 266 bp RNA probe was generated by PCR amplification from the *Acc*I-AlwNI plasmid. M13 Forward and *Tie* 2168 primers (marked in Figure 2) were used for amplification. The probes were labeled using T7 polymerase and [g-32P]-UTP. 10g of poly A(+) RNA was incubated with labelled probe at 50°C overnight. Unhybridized RNA was digested with RNAse A (10 U/ml) and T1 (1 g/ml) at 37°C, pH 7.5 for 1 hour. The RNAses were inactivated by proteinase K digestion at 37°C for 15 minutes and the samples were analyzed in 8% sequencing gels.

The primer extension and RNase protection products terminated at positions 101 bp and 116 bp upstream from ATG codon, in mouse and human *Tie* promoters, respectively (see asterisks in Figure 2). Yeast tRNA or NIH 3T3 RNA did not show any specific bands. Results are shown in Figure 2, wherein the sequences of primers referred to above are underlined.

EXAMPLE III

Construction of plasmids

Tie promoter/luciferase gene constructs were generated by subcloning the 5' flanking 735 bp

genomic *Afl*II-*Apa*I fragment located upstream of the *Apa*I restriction site in the first exon to the promoterless basic pGL2 vector (Stratagene) as described by deWet, *et al.*, *Mol. Cell. Biol.*, 7: 725-737 (1987), incorporated by reference herein, resulting in plasmid 0.73mpromGL2.

For experiments in transgenic mice, the 735 bp *Afl*II-*Apa*I promoter fragment (shown in Figure 2) was blunt-end ligated into a blunted, unique *Hind*III site in the SDK-LacZ Bluescript vector (Stratagene), as described in Logan, *et al.*, *Development, 117*: 905-916 (1993), incorporated by reference herein, resulting in vector 0.73mpromSDK-LacZ. Similarly the 5 kb AlwNI fragment of the human *Tie* promoter shown in Figure 2 was blunt-end ligated into that same vector, resulting in plasmid 5.0hTIEpromSDK-LacZ and deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, as Accession Number 75893

EXAMPLE IV

DNA transfection and preparation of cell lysates

15 ug of the 0.73mpromGL2 plasmid described above was transfected into either LE II mouse lung endothelial cells which are described in Schrieber, *et al.*, *Proc. Natl. Acad. Sci. (USA)*, *82*: 6138-6142 (1985), incorporated by reference herein, or MK-2 cells described by Weissman, *et al.*, *Cell, 32*: 599-606 (1983), incorporated by reference herein. Transfection was accomplished using the modified calcium phosphate mediated transfection method reported in Sambrook, *et al.* (eds.), *Molecular Cloning: A Laboratory Manual (1989)* incorporated by reference herein. The DNAs were

mixed with 0.25 M $CaCl_2$ and an equal volume of 50 mM N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid-buffered saline. The mixture was incubated for 15 minutes at room temperature and then added dropwise on growing cell monolayers.

The resulting cultures were incubated for 12 hours, after which 5% glycerol in PBS (phosphate buffered saline) was added for 30 seconds and washed off with two changes of PBS. Fresh medium was then added. After further incubation for 24 hours, the cells were lysed in 0.5 ml lysis buffer (25 mM Tris-PO4, 2 mM dithiothreitol (DTT), 2 mM 1,2-diamino-cyclohexane, N,N,N',N'-tetracetic acid, 10% glycerol, 1% Triton X-100, pH 7.8). The resulting lysates were centrifuged and the supernatants were collected and stored at 70°C until further assayed. Normalization of luciferase values relative to transfection efficiency was achieved by cotransfection of a CMV-b-gal vector described in MacGregor and Caskey, Nucl. Acids. Res., 17: 2365 (1989), incorporated by reference herein.

Assays for β-galactosidase and luciferase were conducted on transfected cells. For the β-galactosidase assay, 30 ml of the cell lysate described above was incubated in 33 ml of o-nitrophenyl-β-D-galactopyranoside (4 mg/ml) dissolved in 100 ml 0.1 M sodium phosphate, pH 7.5 for 30 minutes at 37°C. Optical density was measured at 414 nm.

Luciferase assays were performed using a FlyLight monitoring Kit (102-100, BioTools, Finland) according to the manufacturer's protocol. Briefly, 20 ml of cell lysate was incubated in 100 ml reaction mixture and a Bio-Orbit 1253 luminometer was used to determine light intensity.

The activity of the *Tie* promoter in cultured cells was measured using *Tie* promoter-luciferase constructs described above. The *Tie* promoter-luciferase constructs were transfected into either LEII endothelial cells or into MK-2 epithelial cells. Promoter activity was determined as the ratio of luciferase to β-galactosidase activity. Those activities were compared to the promoter activity of the positive control vector, RSV-luc (ATCC).

Activity of the 0.73mpromGL2 plasmid (735) relative to CMV-b-gal, used as a constitutively expressed cotransfected control promoter, is shown in Figure 3, along with values for the highly expressed RSV-luc promoter. A 460 bp mouse *Tie* promoter fragment was used in reverse orientation as a negative control (reverse). As shown in figure 3, the *Tie* promoter was highly active in LEII cells but not in the epithelial cells, MK-2. Those results indicate that the isolated *Tie* promoter is specific for vascular endothelial cells and efficiently promotes the expression of the reporter in those cells in comparison to the control.

EXAMPLE V

Production of transgenic mice

The *Tie*-containing transgene was separated from the vector sequence by digestion with *SalI*, purified by electrophoresis through an agarose gel, and recovered by absorption on glass beads (Gene Clean II, Bio 101 Inc., La Jolla, CA) according to the manufacturer's instructions. Transgenic mice were produced by the standard microinjection technique reported in Hogan *et al.*, *Manipulating the*

mouse embryo (Cold Spring Harbor, 1986), incorporated herein by reference. Zygotes for microinjections were obtained from superovulated (BALB/c X DBA/2)F1 hybrid female mice (CD2F1) mated with CD2F1 males. Alternatively, eggs used for injection were from randomly bred superovulated CD1 females. After microinjection, zygotes were transferred at the one or two cell stage into oviducts of pseudopregnant foster mothers (CD2F1 mice). Tail samples were taken from mouse pups at three weeks of age and DNA was isolated from the samples by the salt precipitation method of Miller, *et al.*, *Nucl. Acids. Res.*, *16*: 1215 (1988), incorporated by reference herein. The polymerase chain reaction was used to confirm expression of the transgene using the mouse promoter-specific primer, 5'-CTATTGAGAAGGTTTGGAGG3-3'[SEQ ID NO:6], the lacZ vector primer, 5'-GCTCTAGAACTAGTGGATC-3'[SEQ ID NO:7]; the human promoter-specific primer, 5'-GAGACAGGGGATGGGAAAAA-3' [SEQ ID NO:8]; and the lacZ vector primer, 5'-GAAGATCGCACTCCAGCCAG-3' [SEQ ID NO: 9] using a reaction mixture comprising 200 ng DNA (Tail), 10x buffer (2mM MgCl2), 250 nM Primer 2040, 250 nM Primer 1986, 0.2 mM dNTP Mixture, 0.02 U Dynazyme(Finnzymes, Finland), and 50 ml of distilled water, plus 50 ml mineral oil (M-3516; Sigma, USA). The PCR Program consisted of a hot start at 96°C, 2 minutes, with cycling as follows: 96°C 1 minutes, 50°C 2 minutes, 72°C 3 minutes, for 34 Cycles, with the last step delayed 10 minutes.

EXAMPLE VI

Analysis of *Tie*-containing Tissue

Whole mouse embryos were obtained and stained for β-galactosidase activity. Tissue was

transferred into 4% paraformaldehyde in PBS (pH 7.4) and incubated at 4°C for 20 minutes with gentle agitation. Tissue was then washed with PBS and incubated in fresh X-Gal reaction mixture [1 mg/ml 4-chloro-5-bromo-3-indolyl-β-galactoside, 4mM $K_4Fe(CN)$ 6 x $3H_2O$, 2mM $MgCl_2$ in PBS] at 30°C for 1 to 2 days. Then, samples were washed in PBS for 5 hours and transferred to 30% sucrose for storage.

Samples were then embedded in Tissue Tek (Miles, USA) and 15 μm sections were cut on silane-treated slides. Sections were post-fixed in 4% paraformaldehyde for 5 minutes, and washed twice in PBS and once in distilled water. Nuclear fast red was applied as a counterstain.

Results are provided in Figures 5 and 6. Figures 5A-5D show expression of the mouse *Tie* promoter in 9.5 (Figures 5A and 5B) and 11.5 (Figures 5C and 5D) day post coitum mouse embryos. As seen in the figures, activity of the β-galactosidase reporter gene is found in the developing heart (h), branchial vessels (ba), paired dorsal aorta (da), vitelline artery (v), umbilical artery (u), and in capillaries (c) of 9.5 day post coitum embryos. Two days later (figures 5C and 5D), a similar pattern is found with the addition of staining in the mesonephros (m) and the veins of the liver(l).

Figures 6A through 6F show *Tie* promoter activity in 9.5, 11.5, and 13.5 day post coitum embryos. All endothelial cells of the cardiac region are stained, indicating expression under control of the promoter. Staining is observed in lung, but the bronchi are negative. Brain tissue of 15.5 day embryos also shows staining. Figure 6D shows that the promoter is expressed in veins of the

liver and Figure 6E shows staining in the developing bone trabeculae. The developing cortex of the kidney shows staining, expression being most prominent in the glomeruli.

To study the promoter activity during development, the 0.73mpromSDK-LacZ and 5.0hpromSDK-LacZ DNAs were injected into fertilized mouse oocytes. Six transgenic mice were obtained, transgenic males were mated with wild-type NMRI females and the offspring (86 for 735 bp fragment and 57 for 5.0 kb fragment) were analyzed on days 7.5 - 17.5 of development. Of the F1 offspring, 40% were positive in LacZ staining, although the embryos showed a variation of the intensity of the reaction color. No staining was seen in 7.5 day post-coital embryos, whereas in 8.5 day post coitum embryos, endothelial cells of the dorsal aorta and forming heart were strongly positive. Certain cells of the head mesenchyme, presumably differentiating angioblasts, showed a faint signal, and the extraembryonic tissues, such as allantois and yolk sac, contained positive vessels.

The complexity of the vascular system increases rapidly in the developing embryo, and in 9.5 day post coitum embryos promoter activity was seen in the above mentioned vessels as well as in the intersomitic arteries. An especially intense staining was seen in the developing ventricles of the heart. In 11.5 day post coital embryos the capillary system is well-developed and therefore the staining associated with large vessels of the embryo and the endocardium was only faintly discerned through the dense network of blue-stained capillaries. The details of vascular system were better visualized in high magnification of tissues

of day 11.5 and 15.5 post coital embryos. That staining pattern corresponds to the expression pattern obtained in *in situ* hybridization. As shown in Figures 4A and 4B, the endocardium of the heart, the veins and the arteries of the head mesenchyme showed LacZ signal. No significant differences were seen in the staining patterns obtained with mouse 753 bp and human 5.0 kb promoter fragments.

In order to determine if the promoter activity of the 735 bp mouse fragment correlates with expression of *Tie* mRNA in adult tissues, various tissue types obtained from 8-week old transgenic mice were stained for b-galactosidase activity. As shown in Figures 7A and 7B, intense staining was observed in lung (figure 7A) and bone marrow (designated bm in Figure 7B). Figure 7B also shows staining in capillaries associated with hair follicles (designated by the arrow in Figure 7B). Slightly less staining was observed in kidney glomeruli (Figure 7C, designated "g") and vessels surrounding the tubuli (Figure 7C, designated by the arrowhead). Figure 7D shows staining in the endocardium. Neither large hepatic vessels (v in Figure 7E) or sinusoidal capillaries (not shown) stained with LacZ in adult mice. However, small vessels surrounding the veins did stain [Arrows in Figure 7(E)]. As shown in Figure 7(F), interstitial capillaries of the brain were stained [Arrowheads in Figure 7(F)]. Similar results were obtained when transgenic mice expressed the 5 kb human *Tie* promoter.

The present invention has been described in terms of its preferred embodiments. Accordingly, the invention should be limited only by the scope of the appended claims.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 5, line 28.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☒

Name of depositary institution

American Type Culture Collection (ATCC)

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive, Rockville, MD 20852, USA

Date of deposit	Accession Number
20 September 1994	ATCC 75892

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☒

In respect of those designations in which a European patent or a patent in Finland or Norway is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or the corresponding information concerning the patent in Finland or Norway or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC and the corresponding regulations in Finland and Norway).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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A. The indications made below relate to the microorganism referred to in the description on page 12, line 19.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection (ATCC)

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive, Rockville, MD 20852, USA

Date of deposit	Accession Number
20 September 1994	ATCC 75893

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☒

In respect of those designations in which a European patent or a patent in Finland or Norway is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or the corresponding information concerning the patent in Finland or Norway or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC and the corresponding regulations in Finland and Norway).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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Indications relating to deposited microorganisms

Continuation to C. ADDITIONAL INDICATIONS

ATCC 75892 and ATCC 75893

When designating Australia, in accordance with regulation 3.25 of the Patents Regulations (Australia Statutory Rules 1991 No. 71), samples of materials deposited in accordance with the Budapest Treaty in relation to this Patent Request are only to be provided before: the patent is granted on the application; or the application has lapsed or been withdrawn or refused; to a person who is: a skilled addressee without an interest in the invention; and nominated by a person who makes a request for the furnishing of those samples.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Helsinki University Licensing Ltd Oy

(ii) TITLE OF INVENTION: Promoter for the Receptor Tyrosine Kinase, TIE

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Oy Jalo Ant-Wuorinen Ab
(B) STREET: Iso Roobertinkatu 4-6 A
(C) CITY: Helsinki
(E) COUNTRY: Finland
(F) ZIP: 00120

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Karvinen, Leena
(C) REFERENCE/DOCKET NUMBER: 28203

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +358 0 648606
(B) TELEFAX: +358 0 640575
(C) TELEX: 123505 jalo sf

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 882 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTTAAGACA TGCAACTCGT CTACGGCTAT ACCACTCTGA ACGCGCCCGA TCTCGGAAGA 60

CATGCAACTC AAATGTAAAT ACAGTAGAAT ATTACTTAGG TAGAAACTCC TGGTGATTTT 120

AAAAGATTGG AAAAGAATAT GAGGAAGAGT TGAATAATGC AAATTCTAGT GTGTGTGCTA 180

CCGAAGTGAA CACTTAATGC ACAGTCTACA GACTAGGACA TTTTATCGTG TGTTGTAAAA 240

TTGGGTAGAA ACTTGTGTTT GTGAAAACTG AGCATTAAAA CCTTACAGAG ACCGTTTCTT 300

GTTTACTTTT GAAAAAAAAA AGAGTCACGT GAGCCTCATT TTGTATTTGT GTGTGTGTGT 360

GTGTGTGTGT CTCCCCTCCT CCCAGCGTGT GTGTGCTGGG AGGAGGGGAG ACCCCAGAAC 420

AATGTCCTGC CTCCAAACCT TCTCAATAGG CGGAACGACT GGCTTCTCCC TTTCCTGTCT 480

CCCGTGCTCC AGCAATGCAG ATGGAAGGGA CCGAAGGGAT GGGAGAGAGA GCCCAACCAT 540

CCCCAGATCT GTCCTTGTCA CAACCTGCCT CCCACCTCTA ATGCCCCCCC TTCCAGAGAC 600

TTCCAGGCCA CACCCATCCC GGGCTTGTGG GGGCTGGACA CGGGAGGACT ACAGGCGACA 660

ACTCTTCCCA CCCTCTCTCC CTGCCACCCC TCCTACCCTA ACCATCATTT CCTCTTCCTC 720

CCCAGCACCG AGGTGCACTG AGCTGGACAG GCTGAACACT CAGACCCACA GCAACTGACC 780

CCGGGCCCAG CTGGCCTTGG CTGGCCCAGG GCAGCTTCCA GAGTATGGTC TGGTGGGGAT 840

CCTCTTTGCT GCTCCCCACT CTTTTCTTGG CCTCTCATGT TG 882

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 935 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGGCAAAATG AATGACACCT GGCAGACAAT AAGCTGAAGC TTTCATTAGC AGCTTAAGCT 60

GAGGACTATC TATGCAACCG ATACTCCCTG TGTGCTCCCC GGGATGGTTA ATGTGAGGCC 120

TTGTGGAGCG ATTGGCACCA AGGAAAGGAA GGACTAAGTC AGAAGTTCAA GTCCCAGCCT 180

TGCCACAGCC TCAGGGTGCC CTCGAGCACA GCAAGCCTCA GTTTTCCCAT CTGTACAATG 240

AGAGAGGTAC ACAAGGTAGA CTCGAAGGCT CTTTGTTGCC AGGGCCCTGT GTTCCTTTGA 300

GTGTATGTGC TTCTCAGGCC CACAGAGGTC CTTTGTGTTT CGTATGTGAA CTGCTCTCTA 360

GGAAACCCAT GTAACTGTCT GTGTCCTGGG GCACATACAT GAGGACTCAT GTGGGCCGTA 420

TTGTGTGTTT GTGCCGGGGG GAGGGGAGAC CCCAGAACAA TGTCCCCCAC CCCACCCCCC 480

TCCTCAATAG GCGAAGCGCA CTGGCTTCCT CCCTTTCCTG CCTCCTGCCT CCTTTGTGCC 540

AGCAAGACTG AGTACTGGAG GGAGACAGGG GATGGGAAAA ATCAGTCCAG CTGTCCCCAG 600

GTCTGCCCTT ACCATAACCT TCCCCCCACC TCAAGTGACT CCTCCCAGGC CACACCCATC 660

CCCAGCCTTG TGGGGGCCAG ATTGGGGGGC CTAGAGGCTC AAAGGCAGAA TGAGTCCTCC 720

CACCCCCTAC CCTGCCACCC CTCCCACCCA AGCCACCTCA TTTCCTCTTC CTCCCCAGCA 780

CCGACCCACA CTGACCAACA CAGGCTGAGC AGTCAGGCCC ACAGCATCTG ACCCCAGGCC 840

CAGCTCGTCC TGGCTGGCCT GGGTCGGCCT CTGGAGTATG GTCTGGCGGG TGCCCCCTTT 900

CTTGCTCCCC ATCCTCTTCT TGGCTTCTCA TGTGG 935

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 131 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Gly Cys Val Lys Asp Cys Pro Gly Cys Leu His Gly Gly Val Cys His
1               5                  10                  15

Asp His Asp Gly Cys Val Cys Pro Pro Gly Phe Thr Gly Thr Arg Cys
            20                  25                  30

Glu Gln Ala Cys Arg Glu Gly Arg Phe Gly Gln Ser Cys Gln Glu Gln
        35                  40                  45

Cys Pro Gly Thr Ala Gly Cys Arg Gly Leu Thr Phe Cys Leu Pro Asp
    50                  55                  60

Pro Tyr Gly Cys Ser Cys Gly Ser Gly Trp Arg Gly Ser Gln Cys Gln
65                  70                  75                  80

Glu Ala Cys Ala Pro Asp His Phe Gly Ala Asp Cys Arg Leu Gln Cys
                85                  90                  95

Gln Cys Gln Asn Gly Gly Thr Cys Asp Arg Phe Ser Gly Cys Val Cys
            100                 105                 110

Pro Ser Gly Trp His Gly Val His Cys Glu Lys Ser Asp Arg Ile Pro
        115                 120                 125

Gln Ile Leu
    130
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCACATGAG AAGCC 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGAGATCTTG GAGTATGGTC TGGCGGGTGC CC 32

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTATTGAGAA GGTTTGGAGG C 21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCTCTAGAAC TAGTGGATC 19

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGACAGGGG ATGGGAAAAA 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAAGATCGCA CTCCAGCCAG 20

CLAIMS

What is claimed is:

1. A promoter for a mouse *Tie* receptor tyrosine kinase, said promoter comprising the nucleic acid sequence shown in SEQ. ID. NO: 1.

2. A portion of the promoter according to claim 1; wherein said portion is capable of promoting expression of an endothelial cell receptor tyrosine kinase.

3. A promoter for a human *Tie* receptor tyrosine kinase, said promoter comprising the nucleic acid sequence shown in SEQ. ID. NO: 2.

4. A portion of the promoter according to claim 3; wherein said portion is capable of promoting expression of an endothelial cell receptor tyrosine kinase.

5. A vector comprising the promoter according to claim 1.

6. A vector comprising the promoter according to claim 2.

7. A host cell comprising the vector according to claim 3.

8. A host cell comprising the vector according to claim 4.

9. A host cell comprising the vector according to claim 5.

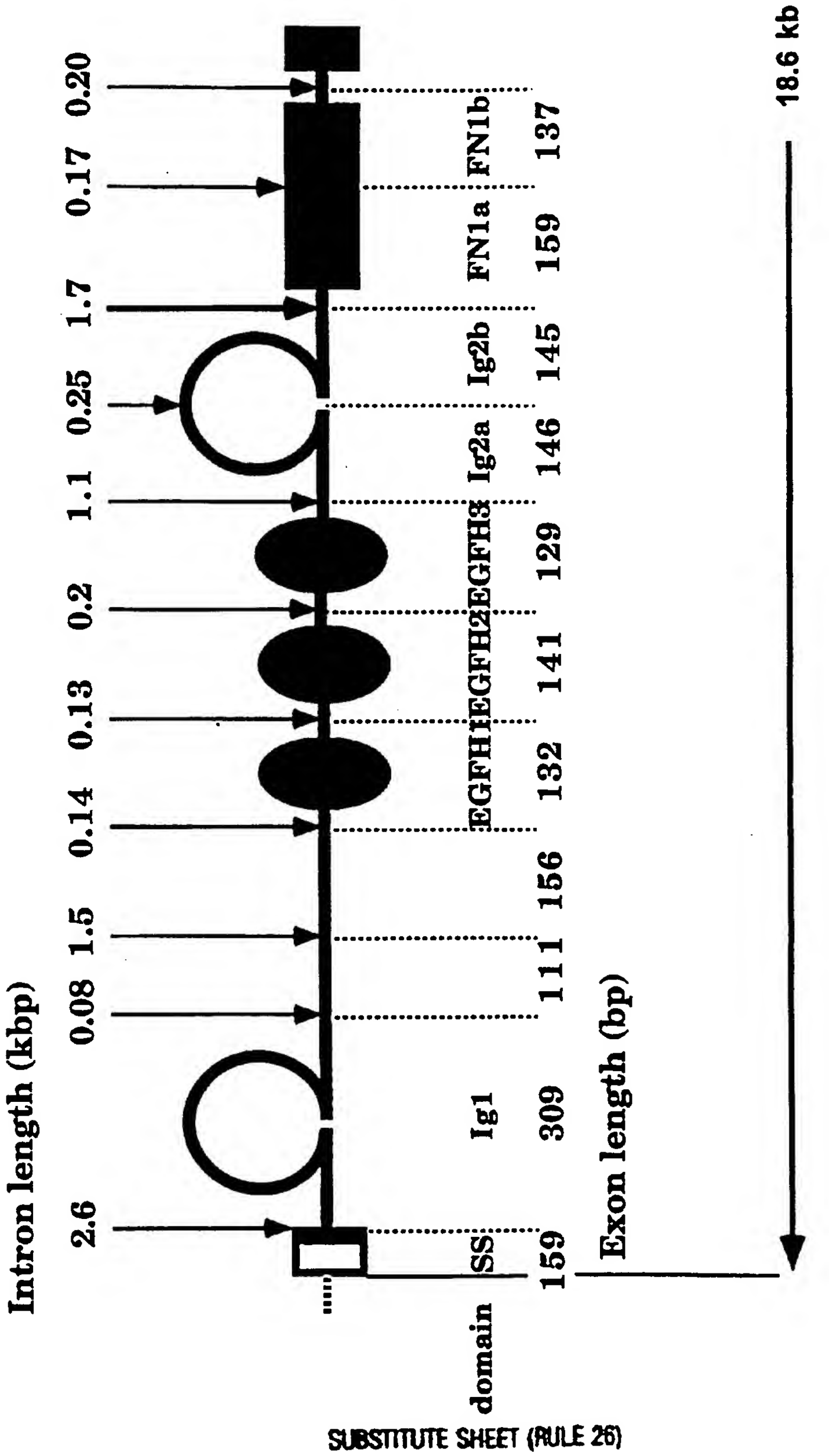
Intron length (kbp)
2.6
0.08
1.5
0.14
0.13
0.2
1.1
0.25
1.7
0.17
0.20
domain
SS
Ig1
EGFHIEGFH2EGFH3
Ig2a
Ig2b
FN1a
FN1b
159
111
156
132
141
129
146
145
159
137
Exon length (bp)
18.6 kb

Figure 1 (1/2)

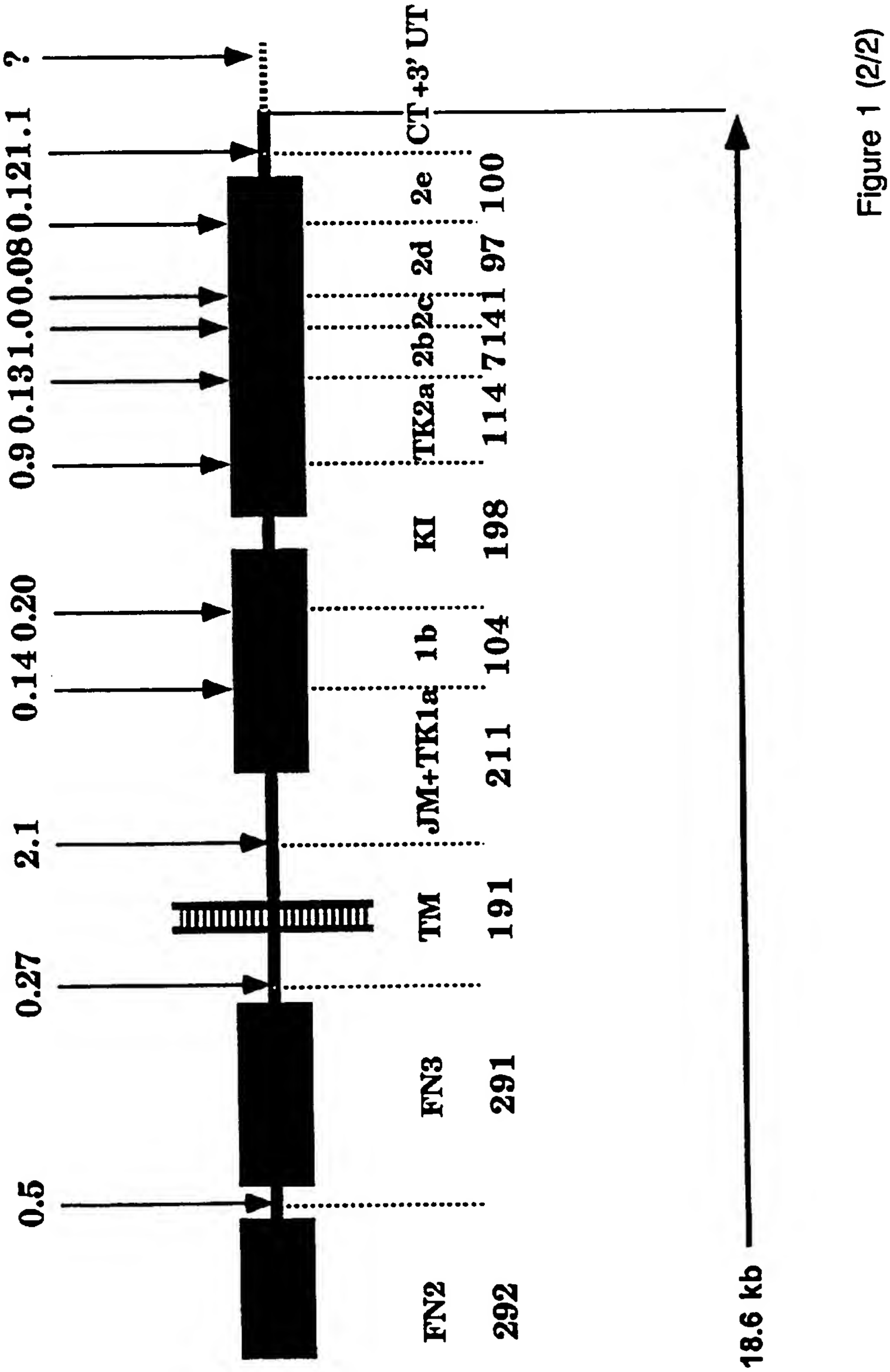
?
0.08 0.12 1.1
0.9 0.13 1.0 0.6
0.14 0.20
2.1
0.27
0.5
CT +3' UT
2e
2d
2c
2b
TK2a
KI
1b
JM+TK1α
TM
FN3
FN2
100
97
141
71
114
198
104
211
191
291
292
18.6 kb

Figure 1 (2/2)

Comparison of mouse and human *TIE* promoter regions

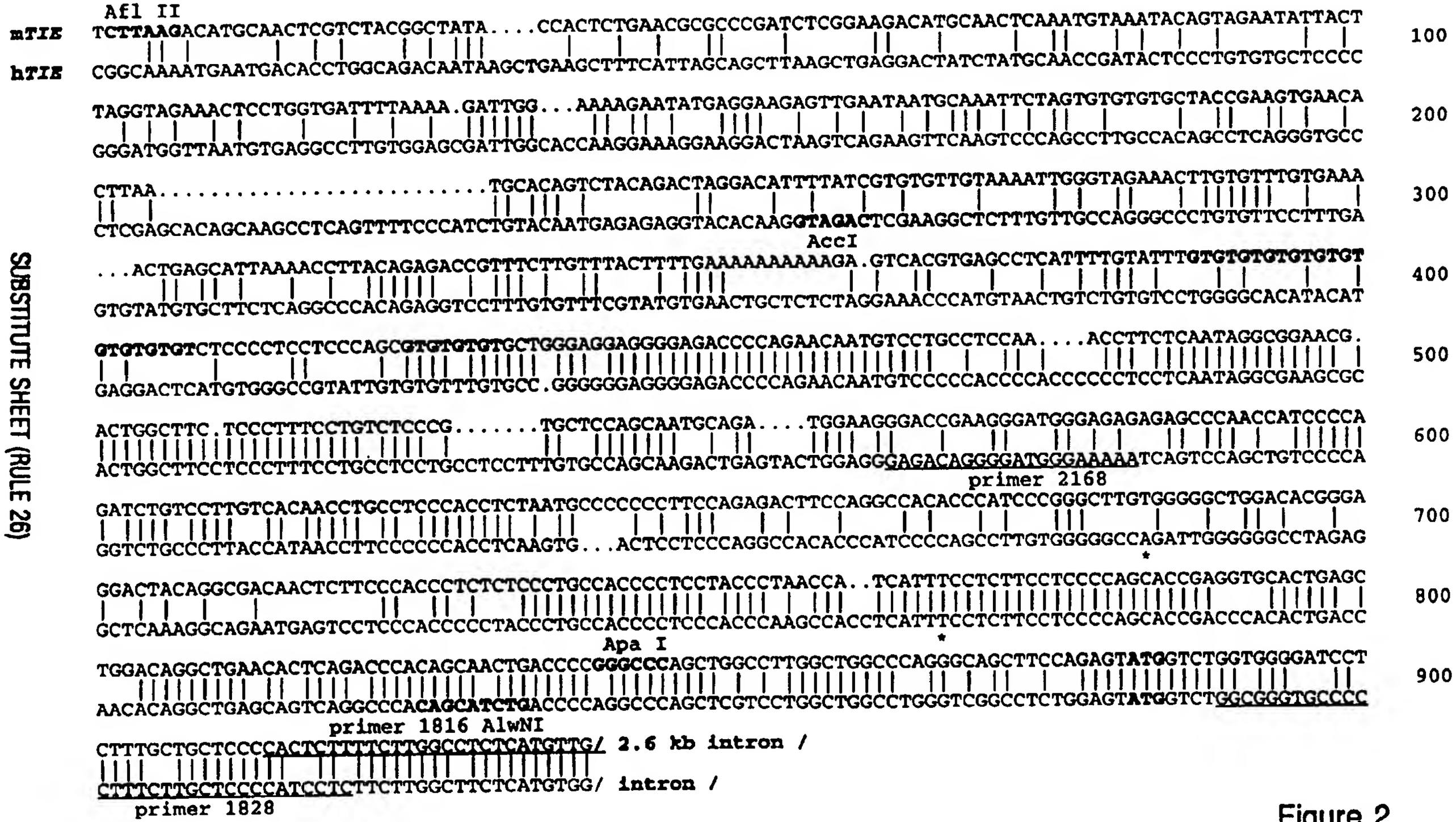

Figure 2

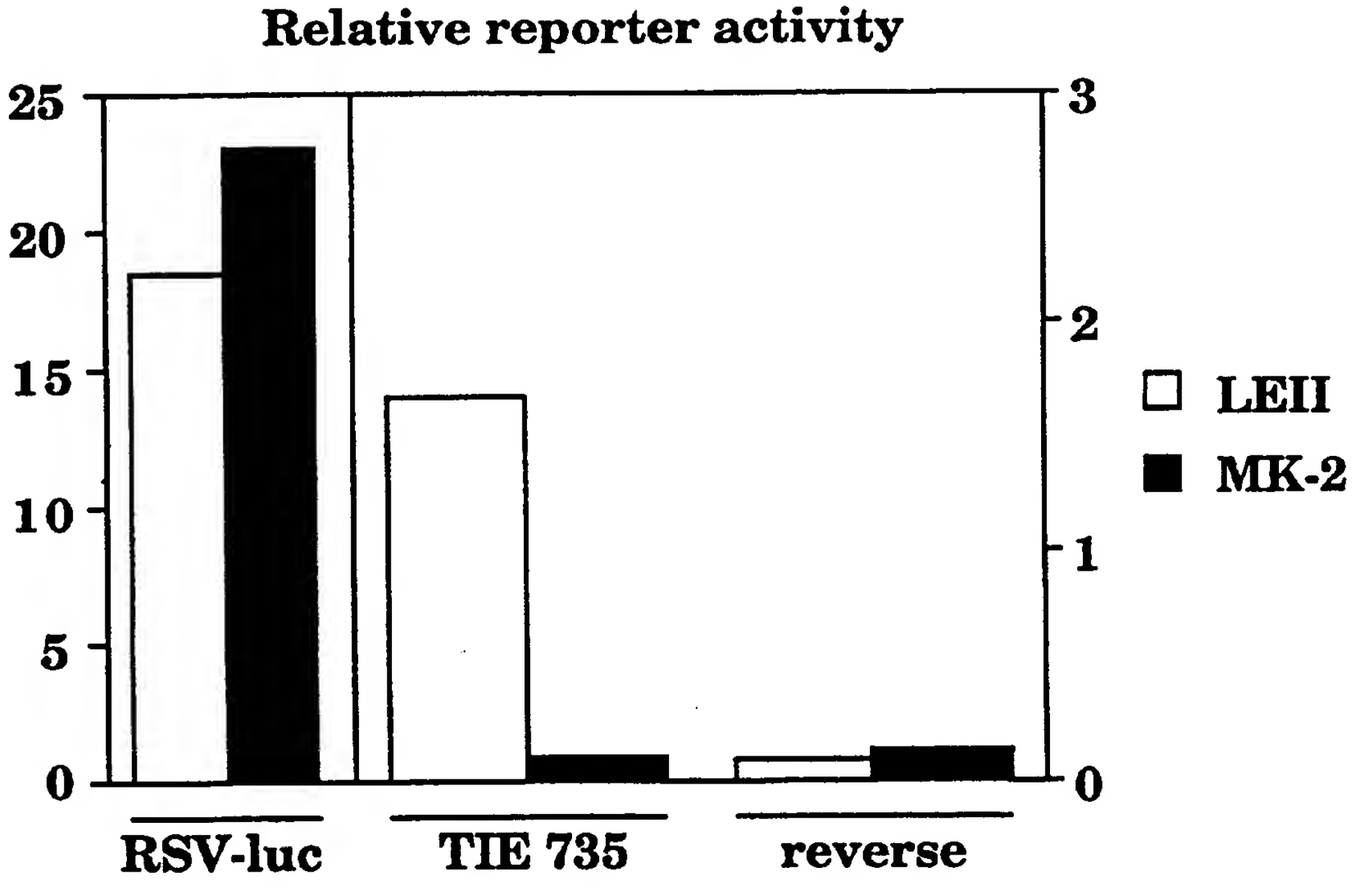
Relative reporter activity
25
20
15
10
5
0
3
2
1
0
LEII
MK-2
RSV-luc
TIE 735
reverse

Figure 3

Figure 4A – 4B

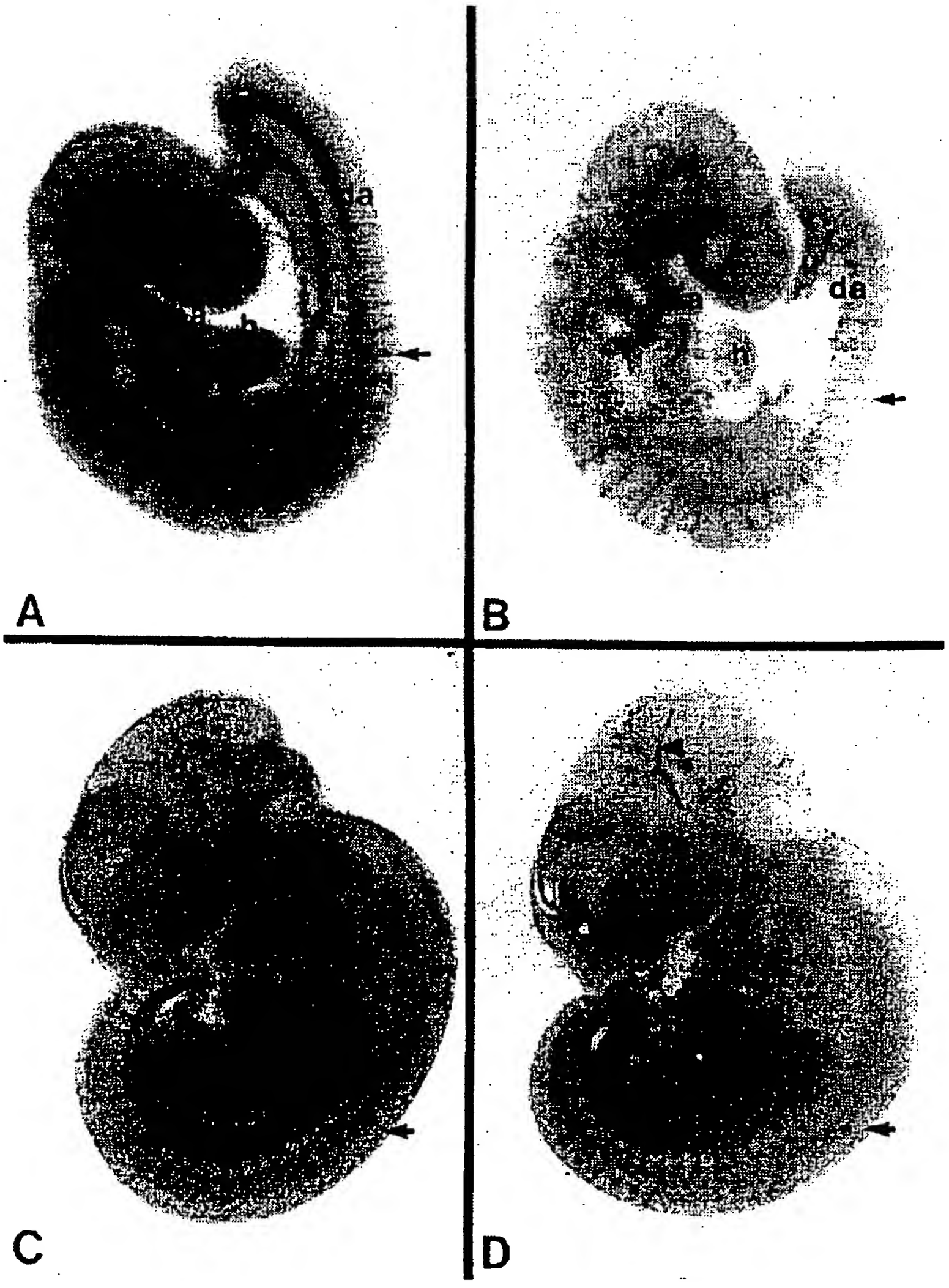

Figure 5A – 5D

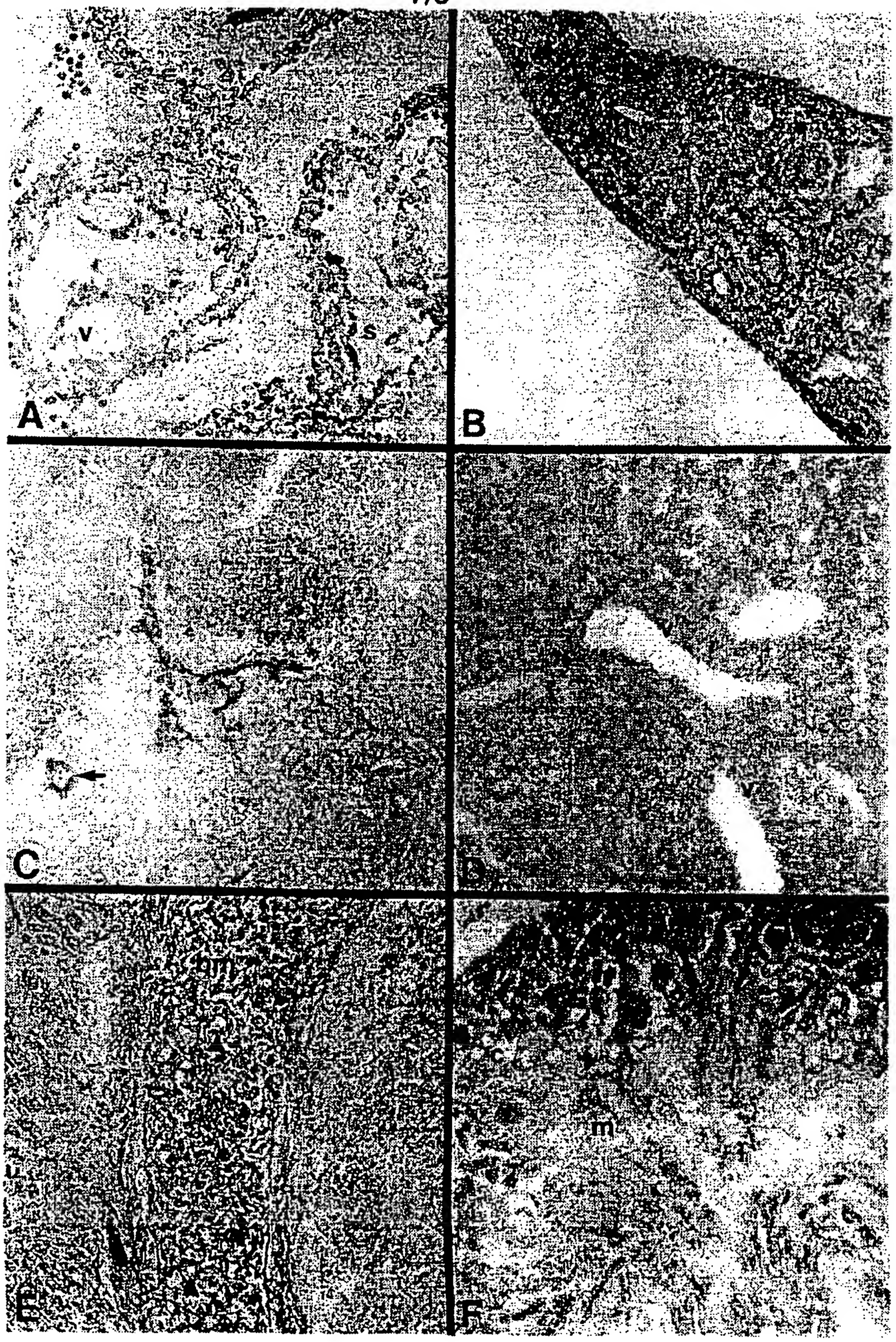

Figure 6A – 6F

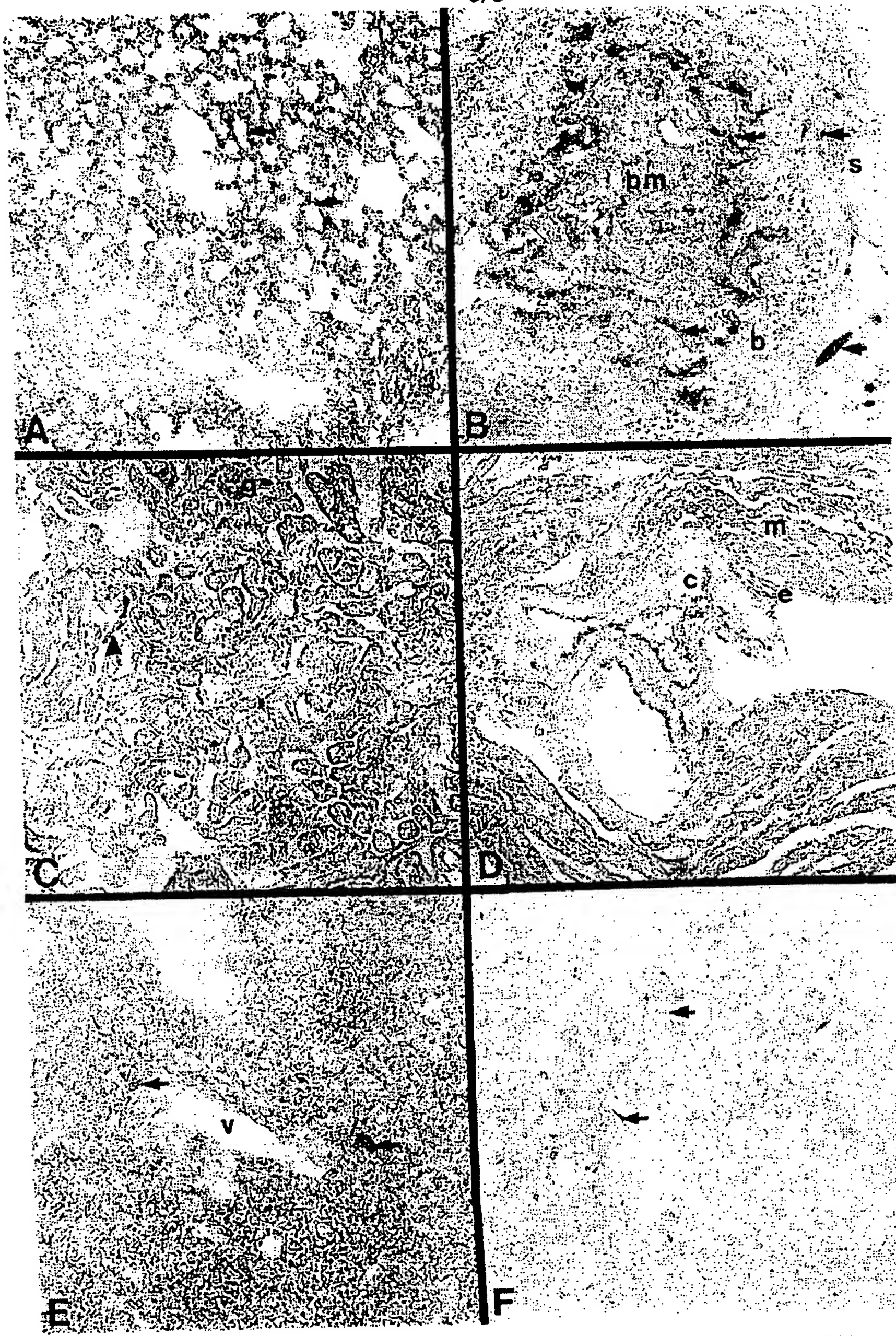

Figure 7A – 7F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/FI 95/00520

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/85 C07K14/71

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BLOOD, SEP 1 1995, 86 (5) P1828-35, UNITED STATES, KORHONEN J ET AL 'Endothelial-specific gene expression directed by the tie gene promoter in vivo.' see the whole document --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 1996	20.02.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nauche, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/FI 95/00520

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, 1993 WASHINGTON US, pages 9355-9358, SATO T.N., QIN Y., KOZAK C.A., ANDUS K.L; 'Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system' cited in the application & EMBL Database EMROD : Mmtie AC=X80764 -08 august 1994- see the whole document	2,6
X	WO,A,93 14124 (HELSINKI UNIVERSITY HOLDING LT) 22 July 1993 see claims 1-11; figure SEQ ID 3	4,8
A	CELL, APR 20 1990, 61 (2) P267-78, UNITED STATES, KERR LD ET AL 'TGF-beta 1 inhibition of transin/stromelysin gene expression is mediated through a Fos binding sequence.'	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/FI 95/00520

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9314124	22-07-93	AU-B-	3353293	03-08-93
		CA-A-	2127540	22-07-93
		EP-A-	0620826	26-10-94
		FI-A-	943275	11-07-94
		HU-A-	69792	28-09-95
		JP-T-	7506242	13-07-95